ANTIMICROBIAL PEPTIDES DERIVED FROM UBIQUICIDINE

The present invention relates to the new medical use of a per se known peptide, which will be referred to as "ubiquicidine" in this application. The invention further relates to new peptide fragments derived from this peptide,

5 optionally in modified form or provided with a (radioactive) label, and the use hereof in prophylaxis, therapy and diagnostics of infections in humans and animals. The invention also relates to new antimicrobial and diagnostic agents on the basis of the peptide, the

10 peptide fragments and/or modified versions thereof, optionally in the form of combination preparations. Finally, the invention also provides a new method for preparing radioactively labelled peptides with antimicrobial activity.

15 In an increasing number of cases the use of what are called "classic" antibiotics is not sufficient for the treatment of infectious diseases. Many bacteria strains have built up resistance against the known classes of antibiotic and in the last thirty years no new classes of

20 antibiotic have been discovered. There are few or no adequate agents against mycobacteria. And other micro-organisms, such as fungi, and determined parasites are also sometimes difficult to treat with existing antimicrobial agents. In view of the above, a new class of

25 antimicrobial agents is highly desirable.
At present, two new types of antimicrobial agents are attracting attention. On the one hand there are the carbohydrate-type agents. In addition, research is focussing on peptides, particularly (cationic) peptides, with

30 antimicrobial activity. Cationic peptides contain a relatively large number of positively charged amino acids, such as arginine and lysine, and therefore carry a net positive charge, usually of at least +2, but often +4 or more. Antimicrobial peptides are an important compo-

35 nent of the natural defence of most living organisms against infections. Many such antimicrobial peptides are

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cationic. In humans and other mammals such peptides, such
as the defensins, are an important protein-like constitu-
ent of for instance neutrophil granulocytes. These cells
are already involved at a very early stage in the defence
5   against micro-organisms and in acute inflammation reac-
tions. In addition, such peptides are also produced by
many other cells, including epithelial cells, which are
strategically located in relation to invading micro-
organisms.

10   In the research which resulted in the present inven-
tion, it was found that the per se known peptide FAU S30
(which has now been called "ubiquicidine" by the present
inventors) has antimicrobial action. It was further found
that peptide (fragments) derived from this peptide also
15   have an antimicrobial action to a lesser or greater
extent. These peptide (fragments) as such have not been
described previously and are therefore still new.

        On the basis of this conclusion, the present inven-
tion provides the use of ubiquicidine or optionally
20   modified peptide (fragments) derived therefrom for pre-
paring a drug for the treatment, diagnostics or prophy-
laxis of infections in humans and animals.

        The advantage of ubiquicidine and fragments thereof
is that they not only have an antimicrobial and immuno-
25   modulating action, but that they also make their way in
the body in targeted manner to the actual site of infec-
tion and accumulate there. These peptide (fragments) are
therefore infection-seeking.

        In this application "antimicrobial action" is under-
30   stood to mean any inhibiting or otherwise negative effect
on bacteria, viruses, protozoa, parasites and fungi.

        "Immunomodulating action" is understood in this
application to mean any stimulating effect on body cells
of humans and/or animals involved in the defence against
35   infections.

        "Ubiquicidine" is understood in this application to
mean a peptide of 6.654 kD with an amino acid sequence as
shown in figure 1.

        Peptide fragments derived from ubiquicidine comprise
40   a preferably continuous series of at least 3, preferably

at least 8 amino acids from the amino acid sequence of
ubiquicidine as shown in figure 1. For an average skilled
person it is simple to ascertain whether a peptide frag-
ment with a series of preferably continuous amino acids
5   chosen from the amino acid sequence of ubiquicidine does
actually have antimicrobial activity and thus meets the
requirements of the invention. A simple standard test for
determining antimicrobial activity is for instance the
universally known growth-killing test, i.e. determining
10 of the concentration of an antimicrobial agent which
kills 99% of the micro-organisms (IC 99%). Designated by
"peptide (fragments)" in this application are therefore
all amino acid chains which are smaller than the ubiqui-
cidine itself, but the amino acid sequence of which is to
15 be found, preferably continuously, in the ubiquicidine.
The length of such peptide (fragments) can vary from 3 to
58 amino acids, wherein possible extra amino acids added
as modification are not included.
        Examples of peptide (fragments) are the peptides of
20 which the sequence is shown in figure 1. Ubiquicidine
(18-35)-D-alanine has as extra addition a D-alanine at
both ends. Of the peptide fragments shown in figure 1,
ubiquicidine (1-18), ubiquicidine (18-35) and ubiquici-
dine (29-41) are particularly recommended. In the above
25 described test the activity of these fragments lies
around 1 μM. This is a particularly good antimicrobial
activity. In principle however, all the above defined
peptides, which display some inhibiting action or other
on micro-organisms, fall within the invention. Peptides
30 with an IC 99% of a maximum of 25 μM, preferably a maxi-
mum of 10 μM, most preferably a maximum of 1 μM are
however recommended.
        In order to modify their activity, for instance to
further increase it, or to inhibit or prevent degradation
35 by enzymes, particularly peptidases, both the peptide
(ubiquicidine) and the fragments can be modified in
different ways. Modification is any variation from the
naturally occurring amino acid chain. Modifications may
be mutual linking in reverse sequence of at least a part
40 of the amino acids of the peptide or a peptide fragment.

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When all amino acids of a peptide (fragment) are thus
reversed, this is referred to as "reverse peptide (frag-
ment)".

5   One or more of the amino acids from the original
peptide (fragment) can also be replaced by a stereoisomer
of that amino acid. The L-isomers of amino acids occur in
the body. The D-stereoisomers can be degraded much less
easily by enzymes present in the body and bacterial
enzymes. Such a modification ensures that the peptide
10   (fragment) in the body remains intact longer and can
exert its effect longer. A similar modification consists
of extending the original amino acid chain at one or both
ends with one or more groups protecting against degrada-
tion, such as D-amino acids, for instance D-alanine.
15   All the amino acid chains modified in the above
described manner or varying in other manner from the
corresponding native peptide (fragment) will be designat-
ed in this application with the term "derivative". These
can be derivatives of the ubiquicidine as well as of
20   fragments thereof.

The invention further relates to so-called "hybrid
molecules", which comprise a (cationic) peptide with an
antimicrobial action and/or a peptide fragment and/or a
derivative thereof according to the invention together
25   with one or more effector molecules. The effector mole-
cule can assume different forms, such as an amino acid
chain, which is capable of binding to a micro-organism
and/or substances secreted by micro-organisms or ex-
pressed on the surface thereof. An example of such an
30   effector molecule is an endotoxin-binding peptide.

Another type of effector molecule can consist of a
virus protein. Such a virus protein/antimicrobial peptide
can enter the host cell, in which the micro-organism for
combatting is situated, in the known manner of a virus
35   and the peptide can exert its antimicrobial action there-
in.

The effector molecule can further be a detectable
label, such as a radionuclide, chosen from the group
consisting of technetium 99m (Tc-99m), iodine 123 (I-123)
40   and 131 (I-131), bromine 75 (B-75) and 76 (B-76), lead 203

(Pb-203), gallium 67 (Ga-67) and 68 (Ga-68), arsenic 72
(As-72), indium 111 (In-111), 113m (In-113m) and 114m
(In-114m), ruthenium 97 (Ru-97), copper 62 (Cu-62), 64
(Cu-64) and 67 (Cu-67), iron 52 (Fe-52), manganese 52m

5   (Mn-52m), chromium 51 (Cr-51), rhenium 186 (Re-186) and
188 (Re-188), terbium 161 (Tb-161) and yttrium 90 (Y-90).
The radionuclide (also called "emitter") can also fulfil
a curative function. Paramagnetic labels, such as fluo-
rine 19 (F-19), sodium 23 (Na-23), phosphorus 31 (P-31),

10   gadolinium 157 (Gd-157), manganese 55 (Mn-55), dysprosium
162 (Dy-162), chromium 52 (Cr-52) and iron 56 (Fe-56) can
also be used.

    According to the invention combinations of effector
molecules can likewise be linked to the peptide. An

15   example thereof are a cell-binding peptide and an emit-
ter, wherein the cell-binding peptide and the antimicro-
bial peptide provide targeting of the hybrid molecule to
the site of infection and the antimicrobial peptide and
the emitter provide for treatment or diagnosis.

20   Hybrid molecules of this type which consist of an
antimicrobial peptide, peptide fragment or derivative
thereof and at least one effector molecule have not been
described previously. The "hybrid molecules" according to
the invention are not therefore limited to the ubiquici-

25   dine as antimicrobial peptide, but generally comprise
hybrid molecules comprising a (cationic) peptide with
antimicrobial activity and/or fragments and/or deriva-
tives thereof. Examples of other such antimicrobial
peptides are α- and β-defensins, protegrins, serproci-

30   dins, magainins, PR-39, cecropins and others (Martin et
al. (1995) J. Leukocyte Biol. 58:128-136).

    The invention relates to the variants of the peptide
or fragments thereof comprehensively described above.
These variants as well as the peptide and the fragments

35   can also be designated collectively in this application
as "peptide (fragments)".

    The invention further relates to an antimicrobial
agent comprising as active component ubiquicidine and/or
peptide fragments thereof, derivatives of one of both

40   and/or hybrid molecules containing at least ubiquicidine

or other antimicrobial cationic peptides and/or peptide
fragments thereof and/or derivatives thereof for use in
the diagnostics, prophylaxis, monitoring or therapy of
infections.

5   The antimicrobial agent according to the invention
can contain only the active component or take the form of
a pharmaceutical composition in which one or more other
carriers, diluents and the like are present. The agent
and the composition can have different forms of adminis-
10   tration, such as for instance tablet, pill, capsule,
injection, infusion, suppository, powder, suspension,
solution, spray, emulsion, ointment, aerosol, plaster or
cream and can be used for oral, anal, nasal, vaginal,
intramuscular, subcutaneous, intravenous, intraperitoneal
15   or local (topical) administration or administration by
means of a catheter via natural or artificial body open-
ings. Other very specific examples of forms of adminis-
tration are toothpaste, tooth varnish and catheters
coated with the active compound. These latter have a
20   prophylactic action.

Compositions according to the invention can be
prepared by combining (i.e. mixing, dissolving et cetera)
of the active component(s) with pharmaceutically and
pharmacologically acceptable excipients with a neutral
25   character (such as aqueous or non-aqueous solvents,
stabilizers, emulsifiers, detergents, additives), and
further, where necessary, colorants, aromatic substances
and/or flavourings. The concentration of the active
component(s) in a pharmaceutical composition can vary
30   between 0.001% and 100% (w/v), depending on the nature of
the treatment and the manner of administering. The dose
for administering likewise depends on the manner of
administration and nature of the treatment. For the mouse
for instance a dose of 1 to 10 μg/kg, for instance 4
35   μg/kg body weight, is suitable. The compositions accord-
ing to the invention are suitable for treatment of both
humans and animals.

The invention further relates to the ubiquicidine,
to peptide fragments thereof, to derivatives of one of
40   both and to hybrid molecules containing at least ubiqui-

cidine or other antimicrobial cationic peptides, and/or
peptide fragments thereof and/or derivatives thereof for
use in diagnostics, prophylaxis, therapy or monitoring of
infections.

5   Infections, which can be treated with the agent are
for instance disorders caused by pathogenic Gram-positive
(Staphylococcus aureus, Listeria monocytogenes including
antibiotic-resistant strains of S.aureus (also called
Multidrug-Resistant S.aureus (MRSA))), and Gram-negative
10 ((antibiotic-resistant) Klebsiella pneumoniae, Escherich-
ia coli, enterococci and Salmonella typhimurium) bacte-
ria, micro-organisms difficult to treat such as Mycobac-
terium avium and Mycobacterium fortuitum, fungi such as
Candida albicans, Cryptococcus neoformans and Aspergillus
15 fumigatis, viruses, in particular enveloped viruses, and
parasites, such as Trypanosoma cruzi and Toxoplasma
gondii. The use of the agent is however not limited to
the infections stated here.

        Because the peptide (fragment) according to the
20 invention is infection-seeking, it can be applied very
well in the diagnostics of infections and pathology
related thereto. If provided with a detectable label, for
instance a radioactive label such as technetium 99m, it
is possible for instance by means of scintigraphy to
25 determine some time after administering where in the body
the peptide (fragment) is situated. This will also be the
site where the infection for treatment is situated. Such
a labelled peptide (fragment) therefore has a dual pur-
pose. Not only is demonstrated where the infection is
30 situated, but the peptide (fragment) will also exert an
antibiotic action due to its presence at the site and
thus reduce the infection. In this manner the effect of
the treatment can also be followed by looking at the
localization of the peptide in time. This is called
35 "monitoring".

        Each of the above mentioned radionuclides can in
principle be used. Particularly recommended however is
technetium 99m (^{99m}Tc). The physical half-life of this
radionuclide amounts to 6 hours and, together with the
40 fact that particularly gamma radiation is emitted, this

means a low radiation load for the patient. The relative-
ly short half-life moreover has the clinical advantage
that the examination can be repeated rapidly. In addi-
tion, this radionuclide is readily obtainable via the
5   commercially available Mo-Tc-nuclide-generator.

It is found that peptide (fragments) labelled with
technetium 99m can already be detected after 15 minutes
at the site of the infection. The accumulation of for
instance gallium 67 takes at least 24 hours. Owing to the
10 very rapid localization of peptide (fragments) labelled
with technetium 99m, a rapid diagnosis is possible.
Furthermore, technetium 99m is mainly a γ emitter with a
very small quantity of the much more harmful ß radiation,
so there is a relatively low radiation load for the
15 patient. A more frequent administration is hereby possi-
ble. In addition, it has also been found that labelling
with technetium 99m has no adverse influence on the
action of the peptide (fragment). In laboratory animal
experiments no adverse effects or changed external char-
20 acteristics due to ^{99m}Tc-labelled peptide (fragments) have
been found up to the present. In vitro studies have
moreover shown that very high concentrations of the
antimicrobial peptide (fragments) are not toxic for human
body cells. Particular recommended therefore according to
25 the invention as hybrid molecules are technetium 99m-
labelled cationic peptides and fragments or derivatives
thereof.

The peptide (fragments) according to the present
invention demonstrate the infection itself and thus the
30 location where the micro-organism is situated in the
body. Known image-forming methods for detecting infec-
tions, such as X-ray, echography and the like are aimed
at demonstrating morphological changes which are the
result of an infection. It is very well possible however
35 for the infection itself to have already disappeared,
while the morphological change still exists. In that case
the treatment of the infection with for instance antibi-
otics is simply continued while this is in fact no longer
necessary. It is recommended in principle to cause a
40 treatment with a determined antimicrobial agent to be as

short as possible in respect of the occurrence of resis-
tances or allergies as a result of the agent. The peptide
(fragments) according to the invention are infection-
seeking and therefore make their way to the site of the
5   infection itself and can also be made visible there. As
soon as the infection has disappeared, this is shown by
the fact that the peptide (fragment) no longer accumu-
lates at the site of the (former) infection. The treat-
ment can then be stopped. Using labelled peptides, infec-
10   tions can also be distinguished from inflammation pro-
cesses. Infections occur when the body reacts to the
presence of a foreign living organism. "Inflammation" is
a general name for reactions of the body to foreign
stimuli, such as particles, molecules, but also live
15   bacteria. The peptide only reacts in the case of infec-
tions.

The invention further relates to combination prepa-
rations which, in addition to ubiquicidine and/or a
peptide fragment thereof and/or a derivative thereof
20   and/or a hybrid molecule, contain one or more other
active components. Combinations with "classic" antibiot-
ics or with antiviral or antifungal agents can for in-
stance be envisaged.

The invention further relates to a method for label-
25   ling antimicrobial peptides, particularly cationic pep-
tides, more particularly ubiquicidine and peptides de-
rived therefrom and defensins. Such a method comprises of
placing the peptide for labelling in contact with a
tin(II) salt, a borohydride and a radioactive label in
30   the presence of alkali, as described in Pauwels et al.
(Nucl. Med. Biol. 20, 825-833 (1993)), but wherein the
peptide is modified with MAG3 (mercapto-acetyl glycine-
glycine-glycine). Prior to labelling the modified peptide
is held at about 100°C for 10 minutes. Particularly in
35   the case of small peptides or peptides carrying no sul-
phur groups, the MAG3 modification results in consider-
ably higher labelling efficiencies.

The whole is stirred at a suitable temperature for a
determined time, for instance 1 to 60 minutes, preferably
40   5 to 30 minutes. The temperature depends on the tempera-

ture sensitivity of the peptide, but will usually lie
between room temperature and 40°C, and will preferably be
about 37°C.

The tin(II) salt is preferably tin(II)pyrophosphate.
5   The borohydride is preferably sodium borohydride or
potassium borohydride. The tin(II) salt and the borohyd-
ride are advantageously used in a ratio between 1:1 and
1:10, preferably 1:4 in quantities of respectively 0.5-5
μl and 2-10 μl. In preference 0.1 M sodium hydroxide is
10 used as alkali.

The radioactive label is advantageously ^{99m}Tc-per-
technetate, but ^{186}Re-perrhenate can also be used. Stan-
dard solutions of such radioactive labels are commercial-
ly available. In the method according to the invention
15 0.05-0.5 ml, preferably 0.1 ml of such a solution is
used.

A particularly advantageous manner of preparing
ubiquicidine, and optionally the fragments, derivatives
and hybrid molecules, is by means of transgenic animals.
20 For this purpose the method comprises of transforming an
animal egg-cell with a gene construct which codes for the
ubiquicidine, peptide fragment, derivative or hybrid
molecule, regenerating a transgenic animal from the
transformed egg-cell and isolating the ubiquicidine,
25 peptide fragment, derivative or hybrid molecule from a
tissue or bodily fluid of the animal, for instance milk.
The products can of course also be synthesized.

The present invention will further be elucidated on
the basis of the accompanying examples, which are only
30 given by way of illustration but do not limit the inven-
tion. Reference is made in the examples to the following
figures, in which:
Figure 1   shows the amino acid sequence of ubiquicidine
           and derived peptides
35 Figure 2   shows the antimicrobial effect of ubiquicidine
           in respect of <u>Klebsiella pneumoniae</u> and <u>Staphy-
           lococcus aureus</u>
Figure 3   shows the effect of ubiquicidine (18-35) on
           herpes simplex virus infection of Vero cells

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Figure 4   shows the effect of ubiquicidine (18-35) on
            Mycobacterium fortuitum
Figure 5   shows the effect of ubiquicidine (18-35) and
            ubiquicidine (18-29) on (antibiotic-resistant)
5   Staphylococcus aureus
Figure 6   shows the effect of ubiquicidine (18-35) and D-
            alanine-protected ubiquicidine (18-35) on Kleb-
            siella pneumoniae
Figure 7   shows the speed of ubiquicidine (18-35) and D-
10   alanine-protected ubiquicidine (18-35) with
            which Staphylococcus aureus is eliminated
Figure 8   shows the effect of ubiquicidine (18-35) and D-
            alanine-protected ubiquicidine (18-35) on (an-
            tibiotic-resistant) Staphylococcus aureus
15 Figure 9   shows the effect of D-alanine-protected ubiqui-
            cidine (18-35) on (antibiotic- resistant) Esch-
            erichia coli
Figure 10   is a scintigram of intraperitoneally adminis-
            tered ^{99m}technetium-labelled ubiquicidine (18-
20   35) in a mouse infected with Staphylococcus
            aureus
Figure 11   is a schematic view of the experimental infec-
            tion and treatment of mice
Figure 12   shows the accumulation of ^{99m}technetium-labelled
25   ubiquicidine (18-35), ubiquicidine (1-18),
            defensins and human IgG in the thigh muscle
            infected with Klebsiella pneumoninae
Figure 13   shows the accumulation of 99mTc-labelled ubi-
            quicidine 18-35 in a nidus but not in inflamma-
30   tions
Figure 14   shows the effect of ubiquicidine (18-35), ubi-
            quicidine (1-18) and defensins on an experimen-
            tal infection with Staphylococcus aureus and
            Escherichia coli
35 Figure 15   shows the antimicrobial effect of ubiquicidine
            29-41 and 18-35 and defensin-1 in mice

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EXAMPLES

EXAMPLE 1

Antimicrobial action of ubiquicidine

1. Introduction

5   By means of gel filtration and reverse phase HPLC a
peptide was isolated from the cytosol fraction of murine
RAW 264.7 macrophages activated with interferon γ and
cells of the human H292 bronchial epithelial cell line
stimulated in different ways. The latter could be stimu-
10 lated with bacterial products (endotoxin, lipoteichoinic
acid), phorbol ester, and bronchial pathogens (Haemophi-
lus influenzae, Streptococcus pneumoniae and para-influ-
enza virus 3). The isolated peptide was called ubiquici-
dine.

15

2. Materials and method

2.1. Isolation of ubiquicidine

      The method of isolating ubiquicidine from cytosol
fractions of cells has been previously described for the
20 isolation of antimicrobial proteins from cell lysates and
cell membrane fractions (Hiemstra et al. (1993) In-
fect.Immun. 61:3038-3046). The cells were cultured in
RPMI 1640 medium with antibiotics and 10% heat-inactivat-
ed foetal calf serum. The cells were subsequently har-
25 vested, washed and resuspended in 10 mM sodium phosphate
buffer (pH 7.4) enriched with a cocktail of protease
inhibitors.

      Using nitrogen cavitation, a cell lysate was ob-
tained whereafter by means of ultracentrifugation at
30 27,000xg a membrane fraction and a cytosol fraction were
obtained. The proteins in the cytosol fraction were
extracted using 5% acetic acid and the acid extract was
dialyzed and subsequently placed on a P60 column.

      The fractions originating from this column were
35 tested for antimicrobial activity. The ubiquicidine-
containing fractions were pooled and further separated by
means of HPLC on a C18 column with heptafluorobutyric
acid as "ion pairing molecule" in the eluent. The HPLC
fractions were likewise tested for antimicrobial activity
40 and immunoreactivity using an antiserum against the N-

terminal part of the ubiquicidine. The pooled fractions
contain pure ubiquicidine.

2.2. Biochemical characterization

5   The sequence of the N-terminal amino acids of puri-
fied ubiquicidine was determined by means of automated
Edman degradation and a peptide sequencer 477A equipped
with a PTH amino acid analyzer 120A (Applied Biosystems,
Foster City, CA). The sequence results were subsequently

10   analysed using the GeneWorks software package (Intelli-
genetics, Mountain View, CA). Molecular weight of ubiqui-
cidine was determined using mass spectrometry (laser
desorption time-of-flight mass spectrometry; Lasermat,
Finnigan MAT LTD, Hemel Hempstead, UK). For the immuno-

15   logical identification of ubiquicidine use was made of a
rabbit antiserum specific to the N-terminal part of
ubiquicidine (ubiquicidine 1-18) and Western blotting.

2.3. Tests for antimicrobial activity in vitro

20   Different techniques were used to test for the
antimicrobial activity of ubiquicidine and peptides
derived therefrom. The gel overlay assay and the radial
diffusion assay have been previously described (Hiemstra
et al. (Infect. Immun. 63, 3038-3046 (1993)). In the

25   growth-killing curve determination which was used to
investigate the IC 99% of the peptide, (mid-log or sta-
tionary phase) bacteria (Klebsiella pneumoniae (A) and
Staphylococcus aureus (B) were exposed for 60 minutes at
37°C to increasing concentrations of the ubiquicidine,

30   whereafter the number of living bacteria in the suspen-
sion was determined using microbiological plate tech-
niques (Colony Forming Units, CFU). As negative controls,
bacteria were exposed to peptide 4 (a synthetic peptide
derived from HIV glycoprotein 120), ubiquicidine (18-29)

35   or no peptide.
         The results of such experiments are shown in CFUs in
Figure 2.

40

3. Result

The ubiquicidine is a 6.7 kD ribosomal cationic
peptide with a pI of 12.67. From sequence determination
of the 18 N-terminal amino acids of the isolated peptide,
5   it was found that these corresponded wholly with the N-
terminal part of the S30 part of the expression product
of the Finkel-Biskis-Reilly murine sarcoma associated
ubiquitously expressed (FAU) gene which occurs inter alia
in humans and mice. The molecular weight of the FAU S30
10 and the ubiquicidine were also found to correspond. It is
therefore assumed that it is the same peptide.

From the determination of the in vitro antimicrobial
action of ubiquicidine it was found that the ubiquicidine
can kill micro-organisms very rapidly (< 10 minutes) and
15 effectively (3-4 log reduction). Figure 2 shows mid-log
Klebsiella pneumoniae (A) and Staphylococcus aureaus (B),
which were exposed for 60 minutes at 37°C to increasing
concentrations of purified ubiquicidine in 10 mM sodium
phosphate buffer. In the control incubations the bacteria
20 multiplied a number of times (not shown). The minimal
inhibiting concentration for said micro-organisms was
found to lie between 0.08 and 0.16 μM, 1.5 μM ubiquici-
dine eliminates Klebsiella pneumoniae (A) almost com-
pletely, while the reduction in the number of Staphylo-
25 coccus aureus (B) amounts to 2 log.

EXAMPLE 2
Antimicrobial action of peptide fragments
1. Introduction
30   A number of peptide fragments were derived from the
native ubiquicidine and the antimicrobial activity there-
of was determined.

2. Materials and methods
35 2.1. Production of synthetic peptides
Peptides were prepared using an Abimed AMS multiple
peptide synthesizer and a fixed phase (tentagel AC, a
polymer of polyethylene glycol spacer linked to a poly-
styrene matrix) (de Koster et al. (1995) J. Immunol.
40 Methods 187:179-188). After completion of the synthesis

the peptide was released from the fixed phase using a
trifluoroacetic acid water(19:1) mixture and the peptides
were subsequently precipitated with an ether pentane(1:1)
mixture at 20°C. After centrifugation the obtained pep-

5   tides were dried at 40°C for 15 minutes. The peptides
were subsequently dissolved in 10% acetic acid and con-
centrated by means of vacuum centrifugation. The purity
of the peptides was determined using HPLC. An overview of
the synthesized peptides derived from ubiquicidine is

10   given in Figure 1. The antimicrobial activity of these
peptide fragments was determined as described in Example
1 under 2.3.

2.2. Antimicrobial effect on Herpes Simplex Virus (HSV)

15   HSV was incubated for 60 minutes with increasing
concentrations of the peptide fragment ubiquicidine (18-
35) at 37°C. The virus preparation was subsequently added
to Vero cells in diverse dilutions. After 3 days at 37°C
the cytopathogenic effect of the virus on Vero cells was

20   determined, with finally made it possible for the virus
titre to be calculated. Figure 3 shows the result.

2.3. Antimicrobial effect on Mycobacterium fortuitum
         About 10^6 Mycobacterium fortuitum were incubated for

25   different intervals at 37°C with 14 μM or 52 μM ubiquici-
dine (18-35) and the number of living mycobacteria in the
suspensions was then determined using microbiological
techniques. The result is shown in Figure 4.

30   2.4. Antimicrobial effect on Staphylococcus aureus
         About 10^6 bacteria of multidrug resistant Staphylo-
coccus aureus (MRSA) and antibiotic-sensitive S. aureus
were exposed for 60 minutes at 37°C to different concen-
trations of ubiquicidine (18-35), whereafter the number

35   of living bacteria in the suspensions was determined
microbiologically. As negative control high concentra-
tions of ubiquicidine (18-29), peptide 4 and no peptide
were used. The result is shown in Figure 5.

40

3. Results

Research into the effect of the different peptides
on Klebsiella pneumoniae and Staphylococcus aureus demon-
strated antimicrobial activity of ubiquicidine (1-18),
5   ubiquicidine (18-35) and ubiquicidine (29-41). The other
peptides were found to be considerably less potent or
inactive.

Figure 3 shows the results of the experiment with
HSV. This shows that an increasing concentration of
10   peptide results in a decrease in the virus titre.

Figure 4 shows that ubiquicidine (18-35) kills M.
fortuitum for a period of 3 hours, whereafter the peptide
then shows a bacteriostatic effect. Repeated administra-
tion at 3 and 7 hours after the first dose results in
15   practically complete elimination of the mycobacteria. In
the control incubations M. fortuitum was found to prolif-
erate. In additional control experiments no indication
was found for agglomeration of the mycobacteria due to
ubiquicidine (18-35) (not shown).

20   Figure 5 shows that the peptide fragment ubiquici-
dine (18-35) results in a marked decrease in the number
of CFUs of different Staphylococcus aureus strains.

EXAMPLE 3

25   Modified peptide fragments and their activity

1. Introduction

A number of the peptide (fragments) described in
Example 2 was further modified in different ways by
adding an extra D-alanine at the beginning and/or end as
30   protection against exopeptidase activity. The antimicro-
bial activity of several "derivatives" obtained in this
manner was likewise determined.

2. Materials and methods

35   2.1. Production of modified peptides

D-alanine-protected peptides were prepared as de-
scribed above (Example 2, ad 2.1) in this application.

2.2. Antimicrobial effect on Staphylococcus aureus

Staphylococcus aureus (5x10⁵ bacteria) was exposed
for different periods at 37°C to 7 μM ubiquicidine (18-
35) and D-alanine-protected ubiquicidine (18-35), where-
after the number of living bacteria in the suspension was
5 quantified microbiologically.

In addition, different strains of (multidrug resis-
tant) Staphylococcus aureus were incubated for 60 minutes
at 37°C with increasing concentrations of D-alanine-
protected and unprotected ubiquicidine (18-35), whereaf-
10 ter the number of living bacteria in the different sus-
pensions was determined microbiologically.

2.3. Antimicrobial effect on Klebsiella pneumoniae
About 5x10⁶ Klebsiella pneumoniae were exposed for 60
15 minutes at 37°C to increasing concentrations of ubiquici-
dine (18-35) and D-alanine-protected ubiquicidine (18-35)
and the number of live bacteria was subsequently measured
microbiologically.

20 2.4. Antimicrobial effect on Escherichia coli
About 10⁶ antibiotic-resistant Escherichia coli and
antibiotic-sensitive E. coli (parent strain of the resis-
tant bacteria) were exposed for 60 minutes at 37°C to
increasing concentrations of D-alanine-protected ubiqui-
25 cidine (18-35), whereafter the number of living bacteria
was determined microbiologically.

3. Results
Comparison of antimicrobial activities of the D-
30 alanine-protected and the unprotected ubiquicidine (18-
35) in respect of Klebsiella pneumoniae in vitro showed
that the D-alanine-protected variant is much more potent
in eliminating the bacteria than the unprotected ubiqui-
cidine (18-35) peptide (Figure 6).
35 The maximum killing effect by both variants of
ubiquicidine (ubiquicidine (18-35) and D-alanine-protect-
ed ubiquicidine (18-35)) on Staphylococcus aureus was
achieved within 15 minutes (Figure 7). The speed of
elimination of Staphylococcus aureus bacteria by the two
40 types of ubiquicidine peptide is identical (Figure 7).

18

The results further demonstrated that the D-alanine-
protected ubiquicidine kills (multidrug resistant) <u>Staph-
ylococcus aureus</u> more effectively than the unprotected
variant (Figure 8).

5   Very surprising is the observation that the D-ala-
nine-protected ubiquicidine (18-35) can kill antibiotic-
resistant <u>Escherichia coli</u> much more effectively than the
antibiotic-sensitive parent strain of <u>Escherichia coli</u>
(Figure 9). 1 μM D-alanine-protected ubiquicidine reduces

10   the number of antibiotic-resistant bacteria to below the
detection limit. A comparable antimicrobial effect rela-
tive to the parent strain is only achieved with 14 μM of
the peptide. This data shows that antibiotic-resistant
bacteria can be eliminated very effectively by peptides

15   derived from ubiquicidine.

EXAMPLE 4
<u>Peptide fragments labelled with Technetium 99m</u>
1. Introduction

20   A hybrid molecule was prepared by labelling the
peptide fragments with the emitter ^{99m}Tc. This example
illustrates the manner of labelling according to the
invention.

25   2. Materials and method
Labelling of peptide D (ubiquicidine (18-35)) and
the D-alanine-protected ubiquicidine (18-35) with ^{99m}Tc
was performed using a method according to the invention.
For this purpose 10 μl of a MAG3-derived peptide solution

30   (2 mg/ml in 0.01 M sodium phosphate pH 3.0) was added to
2 μl of a tin(II)pyrophosphate solution (0.5 mg/ml).
Immediately thereafter 4 μl of a 10 mg/ml KBH$_4$ solution
(Sigma Chemical Company, St. Louis, Mo, US) in 0.1 M NaOH
was added. After adding 0.1 ml of a ^{99m}Tc -sodium pertech-

35   netate solution (20 MBq, Mallinckrodt Medical B.V.,
Petten, Netherlands) the mixture was stirred at room
temperature for 30 minutes.
The radiochemical purity of peptides labelled with
^{99m}Tc was determined after precipitation with 20% trichlo-

40   roacetic acid (TCA), instantaneous thin-layer chromatog-

19

raphy (ITLC) and HPLC. Summarizing, this took place by analysing 20 μl of a freshly prepared ^{99m}Tc-defensin-1 or ^{99m}Tc-IgG on a superose 12 column (Pharmacia, Upsala, Sweden), linked to an LKB Bromma HPLC 2249 chromatography

5   pump (LKB, Upsala, Sweden) and an on-line NAI (Tl)-crystal-gamma-detection system (Raytest Steffi, Germany). The buffer which was used for analysing the ^{99m}Tc-labelled compounds was 14 mM sodium phosphate-buffered salt solution (PBS) pH 7.5 with a flow rate of 1 ml/minute. Label-

10 ling yields of ^{99}Tc-labelled peptides were determined after precipitation with 20% TCA, HPLC analysis and ITLC analysis and were respectively more than 90%, more than 90% and more than 95%.

15 **EXAMPLE 5**
Accumulation of the labelled peptide at the site of infection
1. Introduction
    In order to demonstrate that the peptide (fragment)

20 according to the invention is infection-seeking, the localization of ^{99}Tc-labelled peptides (ubiquicidine (18-35), ubiquicidine (1-18) and defensins in addition to IgG as control) was determined using a γ-camera.

25 2. Materials and method
    Mice were infected intramuscularly with about 10^6 Staphylococcus aureus bacteria (ATCC 25923) and subsequently injected intraperitoneally with 25 μg ^{99m}Tc-peptide. Mice were also injected intramuscularly with about

30 1 x 10^8 heat-killed (1 hour, 100°C) S. aureus, 1 μg endotoxin or 100 ng phorbol myristate acetate (PMA) in order to cause sterile inflammations. At different points in time after injection of the peptide the radioactivity was measured in the circulation (heart), determined organs

35 (liver, kidney, bladder and spleen) and in both thigh muscles using a γ-camera. Accumulation of the labelled peptide at the site of infection in the right thigh muscle is shown in Figure 10.

40

3. Results

The results showed a very short half-life of the
peptides in the circulation, i.e. t_{half} < 15 minutes. The
largest part of the injected labelled peptides (> 60%) is
5   removed via the liver, kidneys and bladder, but a part of
the peptides (1-2% of the injected dose) arrives at the
site of infection in the thigh muscle (Figure 10).

EXAMPLE 6
10   <u>Antimicrobial activity in vivo</u>
1. Introduction

The accumulation and antimicrobial activity <u>in vivo</u>
of a number of ubiquicidine fragments was determined.

15   2. Materials and methods
2.1. Infection model

Reference is made to Figure 11 for a schematic view
of the experimental infection and treatment of the mice.
In summary, mice were infected intramuscularly in the
20   right thigh muscle with about 10^6 bacteria and after 5
minutes injected intraperitoneally with about 25 µg
(labelled) peptide. At different points in time after
injection of the peptide the animals were killed and the
right thigh muscle was removed, homogenized, and finally
25   the number of bacteria in the homogenate was determined
using microbiological plate techniques, or accumulation
of the labelled peptide was determined by means of a γ-
camera. This test involved animals which were normal and
immunocompromised (injection with cyclophosphamide,
30   "total body" radiation).

2.2. Infection-seeking effect of peptides according to
the invention

Mice were infected in the right thigh muscle with
35   <u>Klebsiella pneumoniae</u> and 25 µg ^{99m}Tc-labelled ubiquici-
dine (1-18) or ubiquicidine (18-35) was subsequently
injected intraperitoneally. At different points in time
after injection of the peptide the amount of activity in
the right (test) and left (control) thigh muscle of the
40   mouse was measured using a γ-camera. The results are

shown in Figure 12 as a ratio of the values in the right
thigh muscle and the left thigh muscle, i.e. "target to
non-target ratio". For the purpose of comparison the
results for human defensin and IgG are also shown. The
5   target to non-target ratios for infections and sterile
inflammations were also compared (Figure 13).

2.3. Effect of antimicrobial peptides on experimental
infections
10   Mice were infected in the right thigh muscle with
<u>Klebsiella pneumoniae</u> (A) or <u>Staphylococcus aureus</u> (B). 5
minutes later, 25 μg ubiquicidine (18-35) or ubiquicidine
(1-18) was injected intraperitoneally. 24 hours after
administering of the peptide the animals were killed and
15 the number of bacteria in the right thigh muscle was
quantified microbiologically. As positive control, ani-
mals were injected intraperitoneally with human defensin
and as negative control with the solvent of the antimi-
crobial peptides. The result is shown in Figure 14. The
20 mice were also injected with 150 mg cyclophosphamide/kg
body weight. Four days afterwards the animals were in-
fected in the right thigh muscle with <u>K. pneumonia</u> and a
day later different quantities of ubiquicidine (18-35),
ubiquicidine (29-41) or defensin-1 were injected intrave-
25 nously. Twenty-four hours after administering of the
peptide the animals were killed and the number of bacte-
ria in the right thigh muscle was quantified microbiolog-
ically. As control, normal animals were treated in iden-
tical manner. The result is shown in Figure 15.
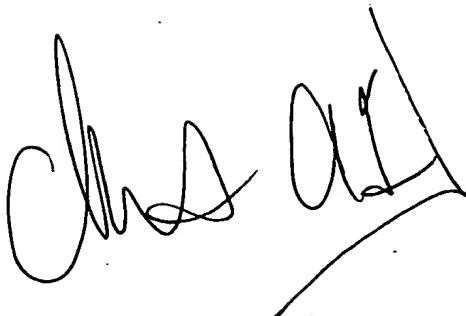30

3. Results
        The accumulation of the tested peptides was found to
be maximal 4 hours after administration and subsequently
decreases in the course of time (Figure 12). It is nota-
35 ble that the maximum accumulation of ^{99}Tc-ubiquicidine (1-
18) and ^{99}Tc-ubiquicidine (18-35) is reached much sooner
than ^{99}Tc-IgG. This observation implies that ^{99}Tc-ubiquici-
dine peptides can be of importance for faster diagnostics
of infections. Comparable results were found when the

^{99}Tc-peptide was administered intravenously 24 hours after infection.

The above stated pharmacological data shows that ubiquicidine (1-18) and ubiquicidine (18-35) accumulate
5   rapidly in the infected thigh muscle. The results of our experiments into the effect of these peptides on the number of bacteria in the muscle demonstrate that ubiquicidine (18-35) eliminates bacteria more effectively than ubiquicidine (1-18) and defensins (Figure 12). These _in_
10   _vivo_ results correspond very well with the results of the _in vitro_ experiments.

Figure 14 shows that particularly ubiquicidine (18-35) also has a marked bactericidal effect _in vivo_ which is better than that of defensin.
15   The result in immunocompromised animals (Figure 15) shows that the bactericidal effect _in vivo_ is determined by a direct bactericidal effect as well as by an immuno-modulating effect.

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